

A Novel Class of MADS Box Genes Is Involved in Ovule Development in Petunia

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We isolated and characterized two ovule-specific MADS box cDNAs from petunia, designated *floral binding protein (fbp)* genes 7 and 11. The putative protein products of these genes have ~90% of their overall amino acid sequence in common. In situ RNA hybridization experiments revealed that both genes are expressed in the center of the developing gynoecium before ovule primordia are visible. At later developmental stages, hybridization signals were observed only in the ovules, suggesting that these genes are involved in ovule formation. To test this hypothesis, we raised transgenic petunia plants in which both *fbp7* and *fbp11* expression was inhibited by cosuppression. In the ovary of these transformants, spaghetti-shaped structures developed in positions normally occupied by ovules. These abnormal structures morphologically and functionally resemble style and stigma tissues. Our results show that these MADS box genes belong to a new class of MADS box genes involved in proper ovule development in petunia.

INTRODUCTION

In higher plants, the female reproductive organ is represented by the pistil, which is commonly composed of the stigma, the style, and the ovary in which the ovules are formed. Inside the ovule, the female gametophyte is produced and surrounded by one or two cell layers, the integuments. After fertilization and embryogenesis, the ovule finally develops into a seed (for review, see Reiser and Fischer, 1993). In spite of the ovule's central role in sexual reproduction of angiosperms, almost nothing is known about the genetic basis of ovule formation and the genes involved in determining ovule identity. In contrast, our knowledge of the molecular processes controlling the formation of the flower that hosts the ovules has increased extensively during the past few years.

After floral induction, the flowering process continues in two distinct steps: the formation of the floral meristem and the determination of floral organ identity. Both steps are controlled by a set of homeotic genes that in part have been isolated and characterized from various plant species. Examples of Arabidopsis genes in the first step are *LEAFY* (Weigel et al., 1992) and *APETALA1* (Mandel et al., 1992). Their homologs from Antirrhinum are *floricaula* (Coen et al., 1990) and *squamosa* (Huijser et al., 1992). In petunia, another homeotic gene, *floral binding protein (fbp)* gene 2, is involved in the determination of floral identity in the central part of the generative meristem. Cosuppression of the *fbp2* gene results in petunia flowers with

no gynoecium and with meristematic cells in the axis of the two carpelloid leaves developing into two new inflorescences (Angenent et al., 1994).

The second set of homeotic genes specifies the identity of the four different floral organs: sepal, petal, stamen, and pistil (from outside to inside). Analysis of a number of homeotic mutants from Arabidopsis and Antirrhinum has led to a genetic model showing how floral organ identity is regulated. In this model, the regulatory genes are subdivided into three classes, A, B, and C. Each is expressed in two adjacent whorls of the flower (for reviews, see Coen and Meyerowitz, 1991; Davies and Schwarz-Sommer, 1994; Weigel and Meyerowitz, 1994). The A function in the first whorl is responsible for sepal formation; expression of class A and B genes together results in petal differentiation; in the third whorl, a combination of class B and C genes determines stamen identity; and the C function alone leads to carpel development. This combinatorial ABC model is supported by molecular data from studies in which the expression of these homeotic genes was altered in transgenic plants from a wide variety of species (for review, see Weigel and Meyerowitz, 1994).

At the moment, no information is available on the molecular control of the formation of the various suborgans and tissues within the floral organs and the way the identity of these structures is established. It is not known how the identity of the ovules formed within the pistil is determined. The existence of homeotic mutants in which ovule identity is changed suggests that homeotic genes distinct from those described above

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participate in the determination of ovule identity. In two tobacco mutants with elevated spermidine levels, one of the pleiotropic alterations observed was a transformation of ovules into style/stigma-like outgrowths (Evans and Malmberg, 1989). The same transformation was observed in transgenic tobacco plants in which the class C gene from oilseed rape (designated *BAG1* for *Brassica AGAMOUS* [AG]) was ectopically expressed under the control of the cauliflower mosaic virus (CaMV) 35S promoter (Mandel et al., 1992). Recently, Modrusan et al. (1994) and Ray et al. (1994) described how, in the *Arabidopsis bell1* (*bel1*) mutant, part of the ovule is also converted into carpel-like structures. This change was accompanied by an up-regulation of the class C homeotic gene *AG*.

Many of the floral homeotic genes encode putative transcription factors sharing a highly conserved region called the MADS box (Schwarz-Sommer et al., 1990). Because it has been shown that MADS box genes play an important role in determining floral organ identity, we hypothesized that there may also be MADS box genes controlling ovule identity. Therefore, we screened petunia pistil and ovule cDNA libraries searching for MADS box genes that are preferentially expressed in the fourth floral whorl. In this study, we describe the characterization of two MADS box cDNAs, *fbp7* and *fbp11*, that are exclusively expressed in ovules. Inhibition of both genes in transgenic petunia plants resulted in a highly specific homeotic transformation of ovules into elongated structures resembling styles and stigmas. The results presented here indicate that the petunia genes *fbp7* and *fbp11* belong to a new class of regulatory genes that are specifically expressed in ovules and are necessary for the correct determination of ovule identity.

Table 1. Pistil and Ovule Development in Petunia Line W115

Stage	Description	Maximum Petal Size (mm)
1	Two carpel primordia arise	0.2
2	Carpel primordia fuse at the base	0.3
3	Placenta develops; gynoecium not fused at the top	0.5
4	Gynoecium fuses at top	0.7
5	Style and stigma initiate	0.9
6	Style elongates and ovule primordia arise	1.0
7	Transmitting tissue and papillae form	1.5
8	Megasporocyte and nucellus tissue form	2
9	Ovule primordia become stalked	4
10	Integument growth initiates	8
11	Megaspore functional after two rounds of meiosis	10
12	Integument covers nucellus completely	15
13	Three rounds of mitosis (megagametogenesis)	20
14	Eight-nucleate embryo sac (mature gametophyte)	30
15	Open bud and anthesis	50

RESULTS

Pistil and Ovule Development in Wild-Type Petunia Flowers

In this study on the molecular control of ovule identity, we started with a detailed microscopic description of petunia pistil and ovule development. The various developmental stages were distinguished on morphological criteria and are briefly described in Table 1 and illustrated in Figure 1. The petunia pistil is composed of two completely fused carpels that arise separately from the floral apex in the center of the flower. The two carpel primordia are morphologically distinguishable shortly after the induction of sepal, petal, and stamen primordium formation (stage 1; Figure 1A). The carpel primordia are horseshoe shaped and, soon after their initiation, form a circular structure by a postgenital fusion process (Verbeke, 1992). This primordial cylinder extends, and before it closes at the top, the placenta starts to develop in the center of the flower (stage 3; Figure 1B). During stages 4 and 5 (Figure 1C), the developing gynoecium closes and style formation starts. In the next stages, the style elongates and the transmitting tissue differentiates, forming a tract through which pollen tubes can grow (Figure 1E). Simultaneously, ovule primordia arise from the placental tissue as a dense group of meristematic cells at developmental stage 6 (Figures 1D and 1F). Within this ovule primordium, a single megasporocyte is formed (Figure 1G) from which a seven-cell embryo sac eventually develops, according to the *polygonum*-type of development (for review, see Willemse and van Went, 1984; Reiser and Fischer, 1993). During this process, the ovules become stalked and an integument is initiated at the base of the nucellus (stage 10; Figure 1H). The integument elongates and grows over the nucellus and finally forms the micropyle. At this site, a pollen tube penetrates the ovule to deliver the sperm cell into the embryo sac for the process of double fertilization. A mature ovule is shown in Figure 1I.

Isolation and Sequence of *fbp7* and *fbp11* MADS Box cDNAs

To isolate pistil-specific MADS box genes from petunia, we constructed a cDNA library made from pistil poly(A)⁺ RNA and screened it with a mixed probe containing MADS box sequences of *fbp1* and *fbp2* (Angenent et al., 1992). Nine independent cDNA clones, designated *fbp6* to *fbp14*, were isolated. An ovule-specific cDNA library was also screened with the same MADS box probe. This probe did not yield new MADS box cDNAs. The expression patterns of *fbp6* to *fbp14* were characterized by RNA gel blot analysis (results not shown), and only two genes, *fbp7* and *fbp11*, appeared to be expressed exclusively in the fourth floral whorl. These genes were selected for further analysis.

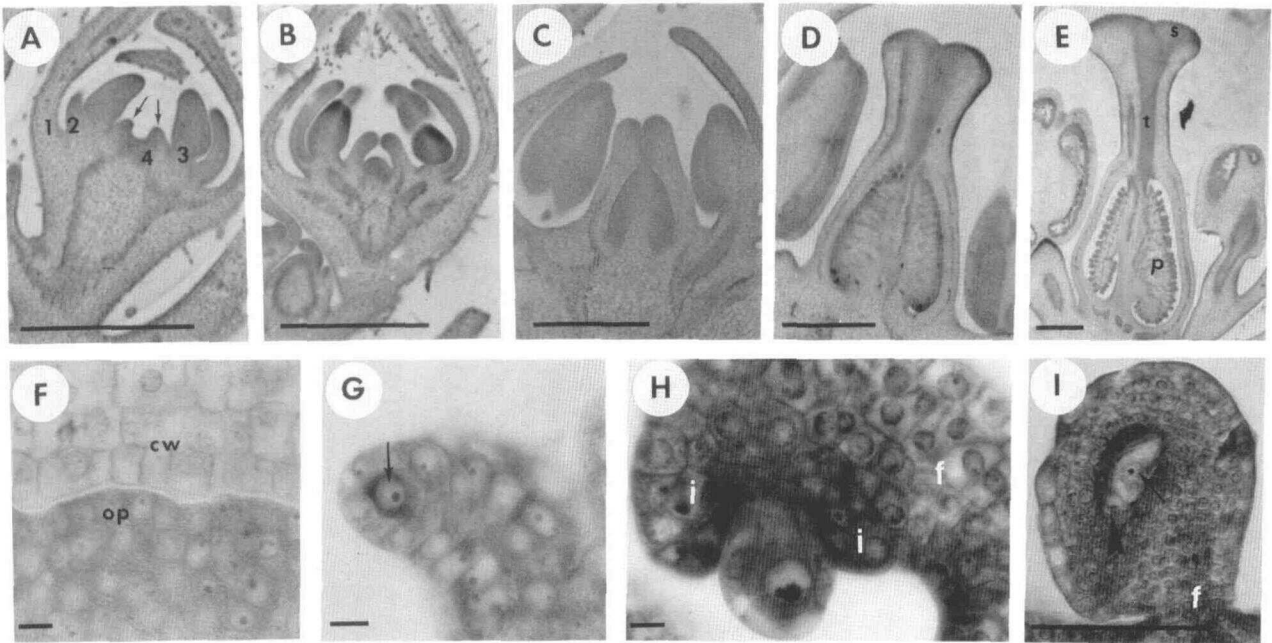


Figure 1. Light Microscopy of Developing *Petunia* Wild-Type Pistils and Ovules.

- (A) Young floral bud with carpel primordia indicated by arrows (stage 1). Floral whorls are indicated by numbers.
 (B) Young floral bud with a stage 3 gynoecium.
 (C) Developing gynoecium at stage 4. The placenta has reached the top of the gynoecium.
 (D) Developing gynoecium at stage 6/7. Ovule primordia and transmitting tissue have formed.
 (E) Pistil at developmental stage 10. Papillae are apparent on top of the stigma.
 (F) Close-up of ovule primordia (stage 6).
 (G) Developing ovule at stage 8. The arrow indicates the megasporeocyte.
 (H) Stage 10 ovule with a premeiotic megasporeocyte within the nucellus.
 (I) Mature ovule with embryo sac. The arrow indicates the central cell with one of the polar nuclei. The arrowhead indicates the egg cell.
 cw, carpel wall; f, funiculus; i, integument; op, ovule primordia; p, placenta; s, stigma; t, transmitting tissue. Bars in (A) to (E) and (I) = 1 mm; bars in (F) to (H) = 0.1 mm.

The complete nucleotide sequence of both cDNAs was determined, and the deduced amino acid sequence is shown in Figure 2. The putative FBP7 and FBP11 proteins have in common 90% of the amino acid residues and share the highest degree of homology (54%) with the *Arabidopsis* AGL1 (for AG-like) and AGL5 proteins (Ma et al., 1991). A homology alignment of the nucleotide sequences of *fbp7* and *fbp11* is depicted in Figure 3A. The full-length *fbp7* and *fbp11* cDNAs are 957 and 1000 bp, respectively, and both contain a complete MADS box region. Extensive nucleotide sequence similarity was observed within the MADS box domain (only 17 mismatches out of 168 bp) as well as outside this conserved region.

To determine the complexity of the *fbp7* and *fbp11* genes in the *Petunia hybrida* genome, we performed DNA gel blot hybridizations under stringent conditions. The results are shown in Figure 3B and indicate that both are single-copy genes. Furthermore, these DNA gel blots demonstrate that the probes used for hybridization are gene specific. Polymerase chain reaction (PCR) analysis using *fbp7*- and *fbp11*-specific

<i>fbp7</i>	1	MGRGKIEIKRIENNTNRQVTFCKRRNGLLKKAYELSVLCE	40
<i>fbp11</i>			D
	41	AEIALIVFSTRGRVVEYSNNNIRAIIDRYKKATVETSNAF	80
		A KGT E A C	
	81	TTQELNAQFYQQESKKLRQIQIQLNSNRHLVGEGLSSLN	120
		A L T A	
	120	VRELKQLENRLERGIARIRSKKHEMILAESEDLQKREIQL	160
		T T N	
	161	EQENAFILRSKIAENERLQELSMMPAGGQEYNAIQQYLARN	200
		T T F F	
	201	MLQLNMME-GVPSY-PLPS--DKKSLDLE	225
		G D AH Q	

Figure 2. Alignment of Deduced Amino Acid Sequence of *fbp7* and *fbp11*.

The complete deduced amino acid sequence of *fbp7* is shown, and differences with the predicted protein sequence of FBP11 are indicated below. The conserved MADS box region is underlined. Dashes denote gaps. The EMBL accession numbers for the *fbp7* and *fbp11* cDNAs are X81651 and X81852, respectively.

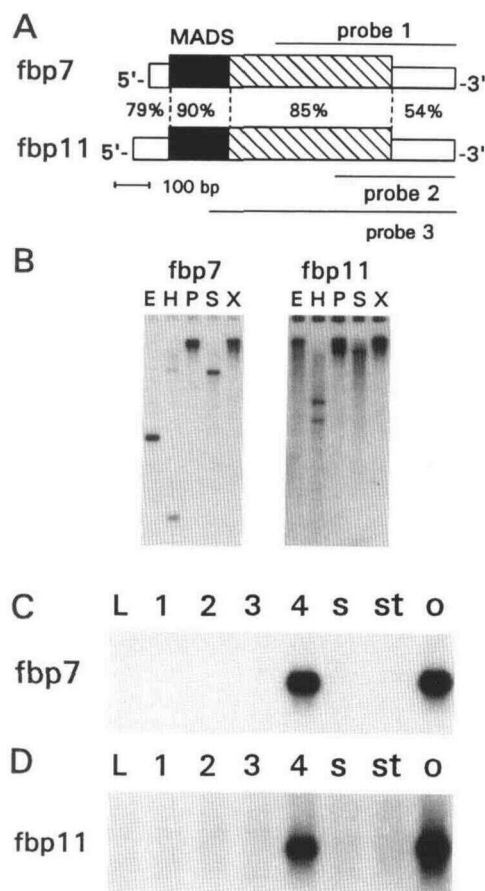


Figure 3. Characterization of the *fbp7* and *fbp11* Gene.

(A) Homology alignment of *fbp7* and *fbp11* cDNAs. Three different domains are indicated. The open boxes are the 5' and 3' untranslated regions; the black boxes are the MADS box regions; the hatched boxes are the coding regions. The percentage of similarity between the domains of *fbp7* and *fbp11* is given. The regions spanned by the probe fragments used for DNA and RNA gel blots in (B), (C), and (D) and RNase protection analysis (see Figure 7) are indicated.

(B) DNA gel blot analysis of petunia W115 genomic DNA hybridized with 3' terminal fragments of the *fbp7* (probe 1) and *fbp11* (probe 2) cDNAs. Ten micrograms of DNA was digested with EcoRI (E), HindIII (H), PstI (P), SacI (S), and XhoI (X).

(C) RNA gel blots with RNA from various plant tissues (petunia line W115) probed with a specific 3' terminal fragment of the *fbp7* cDNA (probe 1). Total RNA was isolated from leaves (L) and mature floral organs and suborgans (lane 1, sepals; lane 2, petals; lane 3, stamens; and lane 4, pistils). s, stigmas; st, styles; o, ovaries.

(D) RNA gel blots similar to the one shown in (C) but probed with a 3' terminal fragment of *fbp11* (probe 2). Lane contents are as given for (C).

primers combined with DNA gel blot analysis revealed that both genes are also present in the two ancestor lines, namely, *P. inflata* and *P. axillaris* (results not shown).

Ovule-Specific Expression of *fbp7* and *fbp11*

The expression patterns of *fbp7* and *fbp11* in wild-type petunia plants (line W115) were analyzed by RNA gel blot analysis using RNA isolated from leaves, the four types of floral organs, and various parts of the mature pistil. Figure 3C shows that *fbp7* was exclusively expressed in the inner floral whorl, where *fbp7* mRNA accumulated in the ovary but not in the style or stigma. A similar hybridization pattern was observed using a 3' terminal cDNA fragment specific for *fbp11* as a probe (Figure 3D).

To determine the tissues and the developmental stages in which these *fbp* genes are expressed, we performed RNA in situ hybridization experiments with developing floral buds. Figure 4A shows a longitudinal section through a young floral bud in which the carpel primordia were just formed (developmental stage 1). No signal was observed in the four floral organ primordia after hybridization with an antisense *fbp11* RNA probe. Expression of *fbp11* was first detectable at the end of stage 3 and the beginning of stage 4, which is just before the two carpels fused at the top. A high uniform hybridization signal was observed in the central meristem from which the placenta and ovules are derived (Figure 4B). No *fbp11* transcripts were present in the carpels or the other floral organs. At later developmental stages, the accumulation of *fbp11* mRNA became restricted to ovule primordia (Figure 4C). Subsequently, when the developing ovule became stalked, *fbp11* was expressed predominantly in the elongating integument and funiculus but not in the nucellus tissue and carpel wall (Figures 4D and 4E). In mature ovules, high levels of *fbp11* transcripts were observed in the endothelium, the cell layer surrounding the embryo sac, whereas little or no signal was detectable in the developing embryo sac (Figure 4F). The same spatial and temporal distribution of the hybridization signals was observed with an *fbp7* probe (data not shown). However, cross-hybridization between *fbp7* and *fbp11* could not be excluded completely in these experiments due to the high homology between *fbp7* and *fbp11* and the conditions used for in situ hybridization. Taken together, the expression data described above demonstrate that both *fbp7* and *fbp11* are ovule-specific MADS box genes with an expression pattern that is clearly associated with the development of the ovule.

Ovule Identity Is Changed by Cosuppression of *fbp7* and *fbp11*

To obtain information about the molecular control of ovule development by the *fbp7* and *fbp11* MADS box genes, we inhibited the expression of these genes by using a cosuppression approach. Cosuppression is a successful method to specifically suppress gene expression in plants, as has been shown for

the chalcone synthase (*chs*) gene (van der Krol et al., 1990) and MADS box genes (Angenent et al., 1993, 1994). The full-length *fbp11* cDNA was fused in the sense orientation behind the CaMV 35S promoter, and this chimeric gene was introduced into petunia line W115 by Agrobacterium-mediated transformation. Three of 40 independent transformants, designated T27017, T27035, and T27040, had ovaries with aberrant ovule development. No aberrations were observed in any other

floral organ or in vegetative tissues. In all three mutants, some of the ovules were replaced by spaghetti-shaped structures (Figure 5B). The remaining ovules were morphologically normal. Due to the shape of the aberrant structures, we have designated these as *spaghetti* mutants. In the primary transformants, these structures were observed predominantly at the top of the ovary or, occasionally, at the bottom or margins of the placenta.

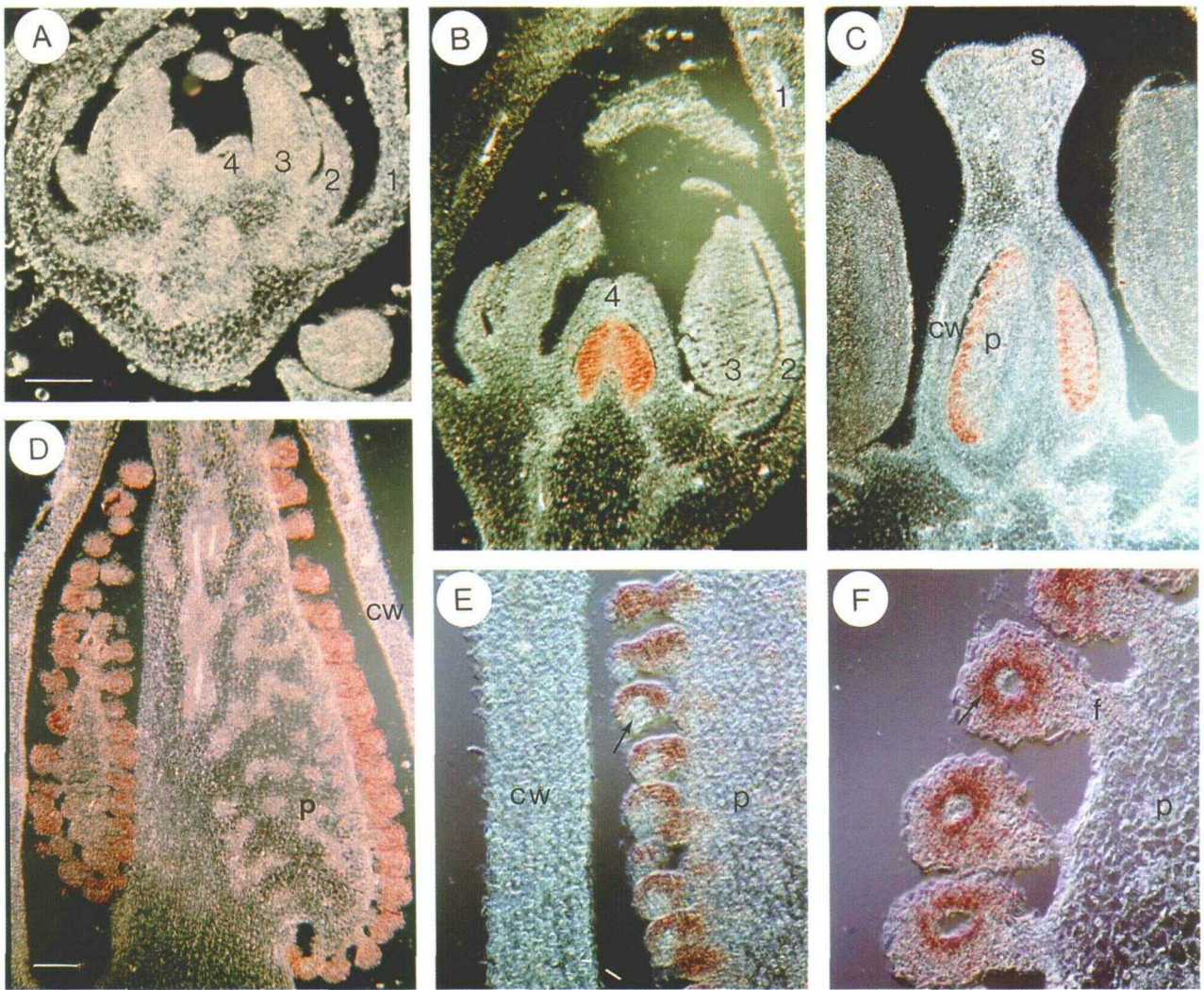


Figure 4. Expression of *fbp11* during Wild-Type Pistil and Ovule Development.

Longitudinal sections were hybridized with digoxigenin-labeled antisense *fbp11* RNA. All sections were viewed using dark-field microscopy. The signal color is red.

- (A) Floral bud with a stage 1 gynoecium. The carpel primordia are formed in the center of the bud.
 (B) Floral bud with a stage 4 gynoecium. The carpel primordia are fused at the top of the developing gynoecium.
 (C) Section through a developing pistil at stage 6 when ovule primordia arise.
 (D) Developing ovary at stage 12.
 (E) Detailed view of stage 10 ovules. Part of the ovary wall and placenta are visible. An arrow indicates the nucellus tissue in which no signal can be seen.
 (F) Almost mature ovules (stage 14) connected with the funiculus to the placenta. The arrow indicates the endothelium cell layer.
 The floral whorls are indicated with numbers. Bars = 0.5 mm. Magnifications are the same in (A) to (C) and in (E) and (F). cw, carpel wall; f, funiculus; p, placenta; s, stigma.

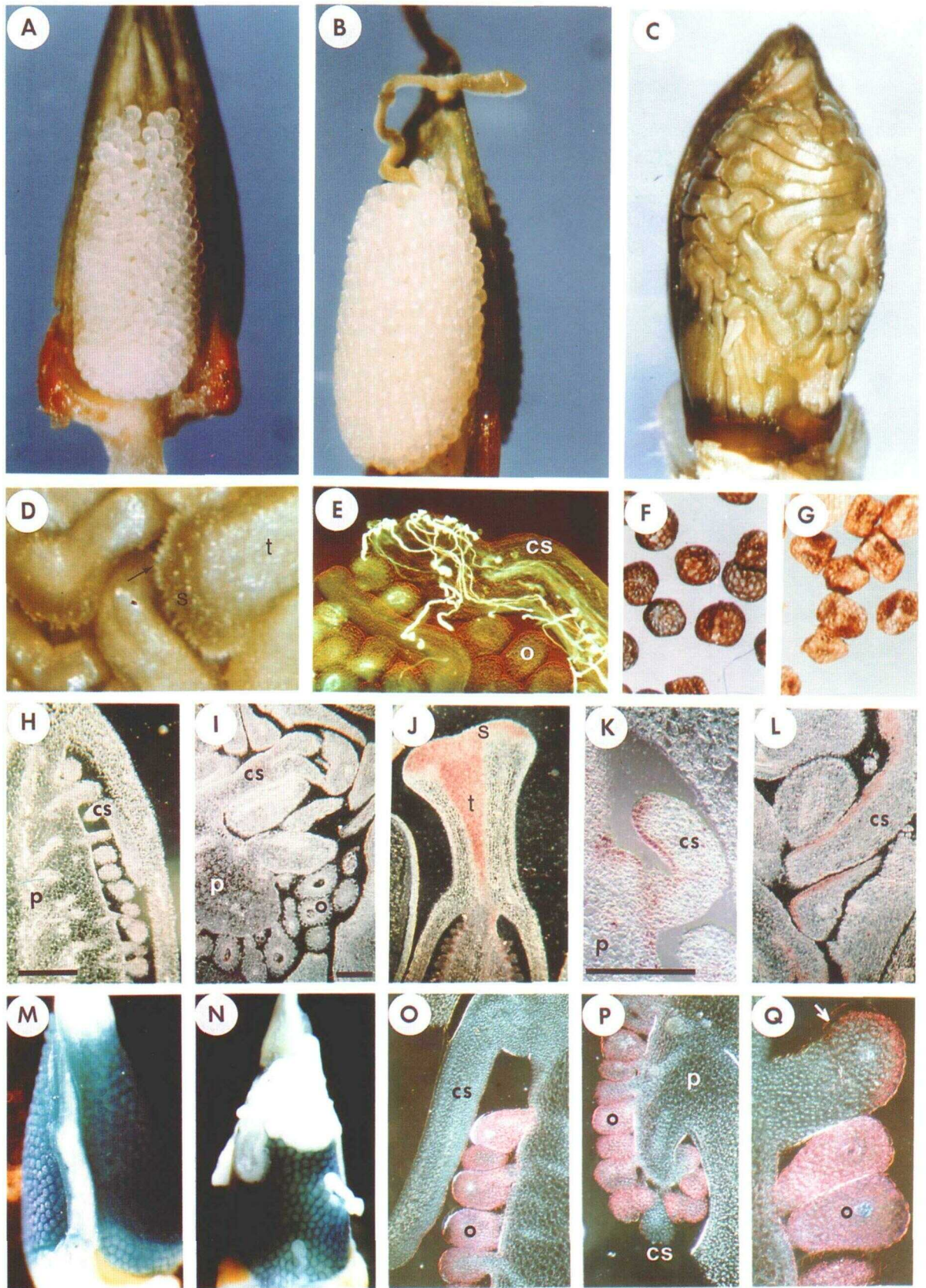


Figure 5. Comparison of the Phenotypes and Gene Expression of the Wild Type and *spaghetti* Mutants.

Two cosuppression plants (T27017 and T27035) were backcrossed with wild-type plants (line W115) to analyze the segregation of the cosuppression trait in progeny plants. Twenty-five plants were analyzed and revealed a ratio of mutant-to-wild-type phenotype of $\sim 1:1$ (11:14 and 13:12 for T27017 and T27035, respectively), indicating that the cosuppression trait is stably inherited in a Mendelian manner. DNA gel blot analysis from these progeny revealed that the mutant phenotype completely cosegregated with one of 13 T-DNA inserts present in the T27017 plant and three of 14 inserts from the T27035 plant (results not shown).

To obtain plants homozygous for the transgenes, the T27017 and T27035 primary transformants were self-pollinated. The seeds obtained from these crosses had an irregular shape compared with the wild-type seed (Figures 5F and 5G), and only a limited number germinated and developed into plants. This suggests that, although the ovules from the *spaghetti* mutants are morphologically normal, their functionality during reproduction was reduced. Three classes were observed in the offspring of the T27017 and T27035 self-pollinations: one with a wild-type phenotype (Figures 5A and 6A), the second with a phenotype similar to the primary transformants (Figure 5B), and a third with ovaries that are completely or almost completely filled with the spaghetti-shaped structures (Figures 5C and 6B). The latter class of plants was designated T27017S and T27035S, and analysis of backcrosses with wild-type plants indicated that these plants are homozygous for the cosuppression trait. No differences in morphology were observed between the carpelloid structures in the primary transformants and in the homozygous progeny plants. Due to the abundance of elongated structures in T27017S and T27035S gynoecia, the ovaries were enlarged two to three times compared with those in the primary transformants and wild-type plants (compare Figures 5A, 5B, and 5C). Only a limited number of morphologically normal ovules were present in the severe *spaghetti* mutants, which did not give rise to any seed set when pollinated with wild-type pollen. In addition to the ovule defects, the size of

the corollas of the T27017S and T27035S flowers was slightly reduced. This might be a secondary effect of the changes in the ovary.

Detailed analysis revealed the presence of papillae on top of the spaghetti-shaped structures; toward the base, elongated cells characteristic of stylar tissue were visible (Figures 5D, 6C, and 6D). To investigate any functional equivalence of these structures with wild-type stigmatic and stylar tissue, we pollinated them with wild-type pollen after partly removing the ovary wall. Pollen germination occurred on the top of the elongated structures, and the pollen tubes grew downward to the base (Figure 5E). No pollen germination was observed on any of the other tissues in the ovary. These observations demonstrate that the spaghetti-shaped structures from the transgenic plants are morphologically and functionally comparable to wild-type carpels.

Ontogeny of the Carpelloid Structures in the *spaghetti* Mutants

The ontogeny of the ovules and carpelloid structures in the severe *spaghetti* mutant (T27017S) was analyzed using light microscopy combined with specific markers for pistil tissues. Differences between T27017S and wild-type ovule development were first discernible at stage 10, at the onset of integument development (Figure 6G). At this stage, normally developing ovules could be observed adjacent to protuberances without any morphologically detectable differentiation into nucellus or integument tissue. These protuberances were first detectable in the upper part of the ovary and originated directly from the placenta at positions normally occupied by ovules (see also Figures 6E and 6F). Shortly afterward (stage 12), cell-dense tissues were formed at one side of the outgrowths (Figure 6H).

To determine the tissue identity of these aberrant outgrowths, we used the expression of *fbp6* (Angenent et al., 1993) as a marker. In situ hybridization experiments revealed that this gene

Figure 5. (continued).

(A) to (C) Ovaries of normal W115 flowers and of mutants T27017 and T27017S, respectively. The ovary walls have been removed. (D) Detailed view of (C). An arrow indicates papillae on top of the carpelloid structures. The transmitting tissue is white, whereas the rest of the carpelloid structure is green. (E) UV microscopic view of pollen tube growth on carpelloid structures in T27017S flowers. The pollen and pollen tubes are white. (F) and (G) Mature seeds obtained from a self-pollination from normal plants (W115) and the *spaghetti* mutant T27017, respectively. (H) to (L) In situ hybridization with digoxigenin-labeled antisense *fbp11* RNA in (H) and (I) and *fbp6* RNA in (J) to (L). All sections were viewed by dark-field microscopy, with the signal being red. Shown in (H) is a longitudinal section through a part of a T27017S ovary (stage 10). (I) shows a cross-section through a mature T27017S ovary. A section with the upper part of a wild-type pistil is shown in (J). Expression of *fbp6* is observed predominantly in stigma and transmitting tissue. In (K), part of a T27017S ovary at stage 10 is shown. The developing carpelloid structure is located at the top of the ovary. (L) shows a cross-section through a mature T27017S ovary. (M) to (Q) *chs*-driven GUS activity in wild-type and T27017 backgrounds. (M) and (N) provide a macroscopic view of mature ovaries from a normal plant (W115) and from a *spaghetti* mutant (T27017), respectively. Before staining for GUS activity, the ovary walls were removed completely. (O) to (Q) show longitudinal sections through a *gus*-expressing *spaghetti* mutant (stage 11). The sections were viewed by dark-field microscopy, with the signal color being red. A structure with a chimeric identity is indicated with an arrow in (Q). Bars = 1 mm. Magnifications are the same in (H), (L), (O), and (P); (I) and (J); (K) and (Q). cs, carpelloid structure; o, ovule; p, placenta; s, stigma; t, transmitting tissue.

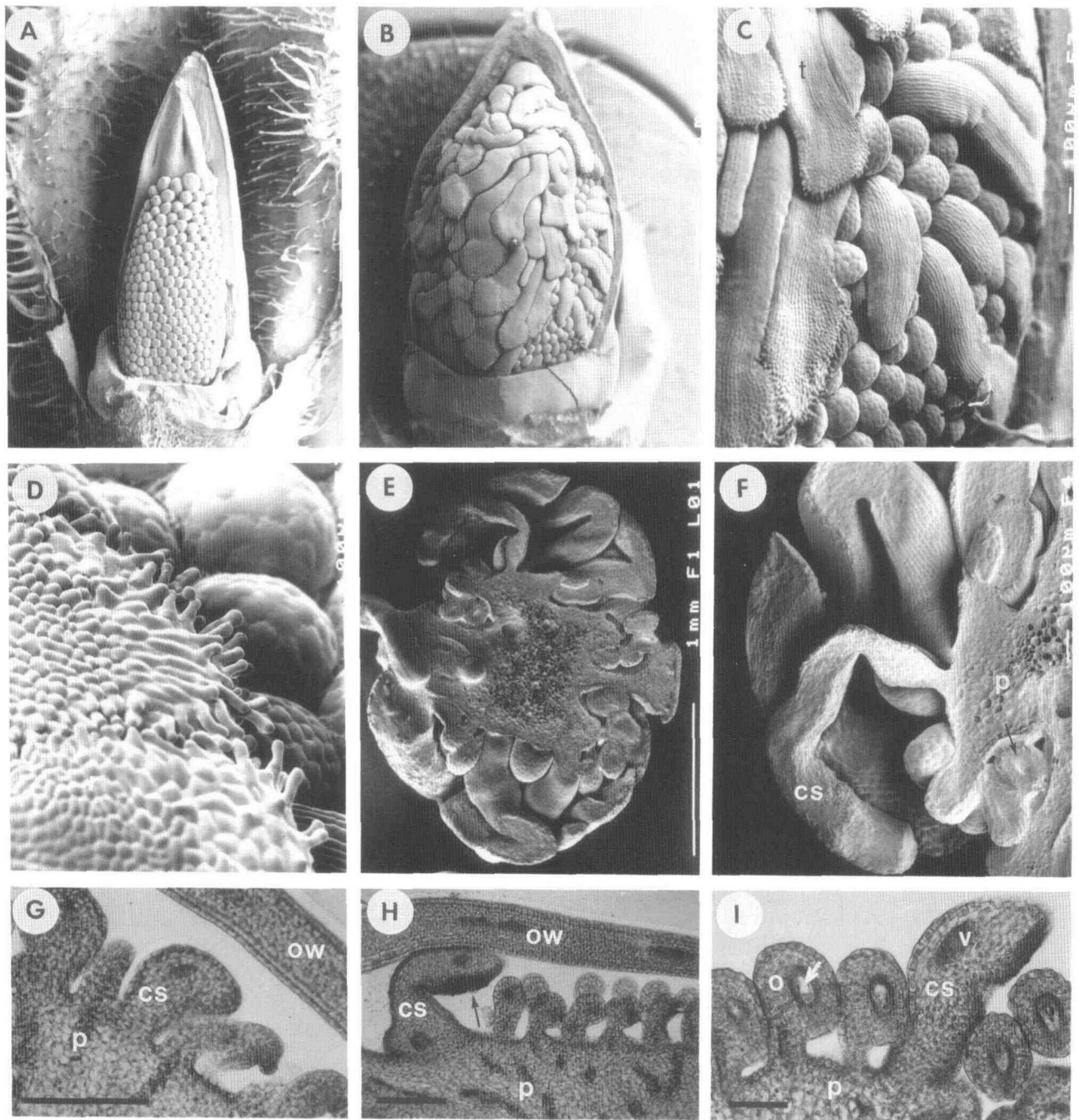


Figure 6. Morphology of the Carpeloid Structures in the *spaghetti* Mutant.

(A) to (F) Scanning electron microscopy of mature ovaries from wild-type and T27017S plants. In (A) and (B), ovaries of a wild-type plant and the *spaghetti* mutant, respectively, are shown. The ovary walls have been removed. (C) is a close-up view of the ovules and carpeloid structures shown in (B). (D) is a close-up view of the stigmatic tissue with papillae shown in (C). In (E), a cross-view through a T27017S ovary is shown. In (F), a close-up of the carpeloid structures and ovules shown in (E) is provided. An ovule is indicated with an arrow. Bars = 1 mm.

(G) to (I) Light microscopy of longitudinal sections of developing carpeloid structures and ovules in T27017S. In (G), a stage 10 ovary in which the carpeloid structures are morphologically distinguishable from the normal ovules is shown. Integument growth has not been initiated yet in the normal ovules. In (H), a stage 12 ovary with a carpeloid structure on top of the ovary is shown. The cell-dense tissue representing developing transmitting tissue is indicated with an arrow. In (I), mature ovules adjacent to a carpeloid structure that originates directly from the placenta is shown. The embryo sac is indicated with an arrow.

Bars in (G) to (I) = 1 mm. cs, carpeloid structures; o, ovules; ow, ovary wall; p, placenta; t, transmitting tissue; v, vascular bundle.

is highly expressed in stigma and transmitting tissue of wild-type flowers (Figure 5J). Figure 5K shows a clear hybridization signal in the cell-dense tissues observed in stage 10 ovaries, indicating that these tissues represent developing transmitting tissue and stigmatic cells. Also in ovaries of mature *spaghetti* mutants, the *fbp6* signal was present in these cell groups, once again showing their carpeloid nature (Figure 5L).

The identity of the morphologically normal ovules in the primary mutant was examined using the activity of the *chs* promoter as a marker. This promoter is highly active in ovules, and expression of the β -glucuronidase (*gus*) reporter gene driven by the *chs* promoter was initiated at stage 10/11 during ovule development (Ylstra et al., 1994). Transgenic plants expressing the *gus* gene driven by the *chs* promoter were crossed with T27017 plants, and the progeny were selected for the *spaghetti* phenotype and GUS activity. Figures 5M to 5Q show GUS activity in the ovaries of plants with wild-type and T27017 backgrounds. Ovules in the T27017 mutant background exhibit a *chs*-driven GUS activity similar to that in wild-type ovules. In contrast, no activity was observed in the aberrant structures present on the bottom and top of the ovary (Figures 5O and 5P), as was expected based on their carpeloid nature. In addition to the carpeloid structures showing no GUS activity, chimeric organs with partial ovule identity were also present (Figure 5Q). The cells with ovule identity were always observed at the top of the chimeric structures and probably represent integumental cells. This is probably due to incomplete cosuppression of *fbp7* and *fbp11* during early stages of ovule development in the primary transformant. Incomplete cosuppression was also suggested by RNase protection experiments (see Figure 7A).

Late during flower development (after stage 14), papillae became evident on the top of the carpeloid structures. At the bases, these structures originated directly from the placenta without any resemblance to the funiculus of normal ovules (see Figures 6F and 6I), demonstrating that the complete ovule is transformed. The development of the morphologically normal ovules proceeded as in wild-type flowers, resulting in mature embryo sacs (Figure 6I).

Specific Down-Regulation of *fbp7* and *fbp11* in the *spaghetti* Mutants

Expression levels of *fbp7* and *fbp11* in wild-type plants and transgenic plants T27017, T27017S, T27035, and T27035S were determined by RNase protection analysis, as shown in Figure 7A. *fbp7*- and *fbp11*-specific fragments were protected by their corresponding mRNAs present in wild-type ovaries. In the ovaries of the T27017 and T27035 primary transformants, the amount of *fbp11* mRNA was found to be reduced dramatically to 5 and 9%, respectively, of the levels found in wild-type ovaries. Surprisingly, the accumulation of *fbp7* mRNA in both transgenic plants was reduced to the same extent as *fbp11*. In the *spaghetti* mutants homozygous for the *fbp11* cosuppression trait (T27017S and T27035S), no mRNA of either gene was detectable. This agrees with the more severe phenotype of these

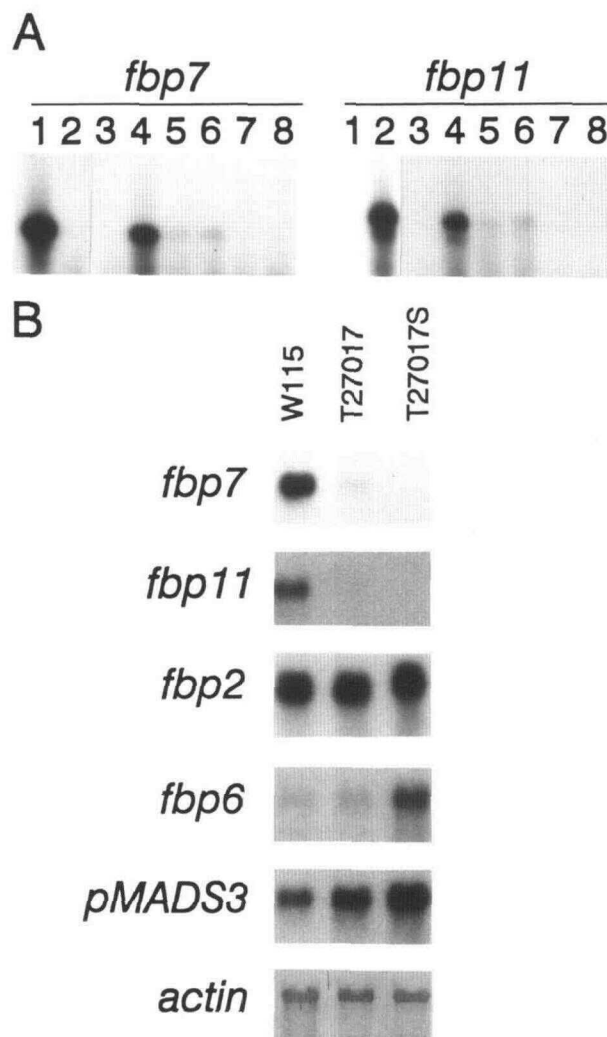


Figure 7. Comparison of Gene Expression in Mature Wild-Type Plants and *spaghetti* Mutants.

(A) Expression of *fbp7* and *fbp11* as determined by RNase protection analysis. RNAs were hybridized with 32 P-labeled antisense RNA transcripts from either *fbp7* (583 bases; see probe 1 in Figure 3A) or *fbp11* (795 bases; see probe 3 in Figure 3A). Labeled transcripts were hybridized with the following: sense *fbp7* transcripts, lanes 1; sense *fbp11* transcripts, lanes 2; petal RNA (W115), lanes 3; RNA from wild-type ovaries, lanes 4; RNA from T27017 ovaries, lanes 5; RNA from T27035 ovaries, lanes 6; RNA from T27017S ovaries, lanes 7; and RNA from T27035S ovaries, lanes 8. All samples were treated with RNases.

(B) Comparison of gene expression in normal petunia (W115) and *spaghetti* mutants T27017 and T27017S by RNA gel blot analysis. Total RNA was isolated from wild-type ovules, T27017 ovules (without carpeloid structures), and carpeloid structures present in T27017S ovaries. Blots were probed with 32 P-labeled 3' terminal fragments of *fbp2*, *fbp6*, *fbp7*, *fbp11*, and *pMADS3* cDNAs. The *pMADS3* blot was rehybridized with the digoxigenin-labeled actin probe as a control for the amount of RNA loaded.

plants, compared with the primary transformants. These data were confirmed by in situ RNA hybridization experiments (Figure 5I) in which no *fbp11* mRNA was detectable in mature ovaries of the *spaghetti* mutant T27017S. Also, at earlier stages of ovule development (stage 10), *fbp11* expression was completely inhibited in T27017S ovules, as demonstrated by RNA in situ hybridization (Figure 5H).

This cosuppression approach appeared to be very specific for *fbp7* and *fbp11* because the expression of *fbp2*, another MADS box gene acting early during flower development, was not affected in the *spaghetti* mutants (Figure 7B). Also, for other cosuppression mutants, we have shown that the MADS box genes *fbp1* and *fbp2* are specifically down-regulated (Angenent et al., 1993, 1994). Nevertheless, it cannot be excluded that other MADS box genes are also cosuppressed in the *spaghetti* mutant.

Expression of C-Type MADS Box Genes in the *spaghetti* Mutants

Because ovules were homeotically transformed into carpels in the *spaghetti* mutant, we were particularly interested in the expression of C-type MADS box genes. The petunia genes *fbp6* (Angenent et al., 1993) and *pMADS3* (Tsuchimoto et al., 1993), which are both very homologous with the Arabidopsis class C gene *AG*, were used in this experiment. To study the expression of *fbp6* and *pMADS3* at a developmental stage in which the fate of the ovule primordia is determined (stage 6), in situ hybridization experiments were performed with developing pistils from wild-type plants (Figures 8A, 8C, and 8E) and the T27017S *spaghetti* mutant (Figures 8B, 8D, and 8F). At this stage, the decision to become an ovule or a carpelloid structure is made. Figure 8B shows that at developmental stage 6, the expression of *fbp11* was completely abolished in a T27017S ovary, which agrees with the results of the RNase protection and RNA gel blot experiments shown in Figure 7. The *fbp6* gene was expressed predominantly in the developing transmitting tissue and ovule primordia (Figure 8C) from wild-type plants. In the severe *spaghetti* mutant, the level of *fbp6* expression in the ovule primordia was comparable with expression levels in wild-type plants (Figure 8D). The developing transmitting tissue was not visible in the longitudinal section shown in Figure 8D, which explains the difference in *fbp6* expression at the top of the ovaries of a wild-type plant and the mutant. Consecutive sections were used for the hybridization with a *pMADS3* probe (Figures 8E and 8F). Also for *pMADS3*, the expression in ovule primordia was not dramatically changed in the *spaghetti* mutant.

The effect of *fbp7* and *fbp11* suppression on C-type gene expression was also monitored by RNA gel blot analysis (Figure 7B). Because the *fbp6* and *pMADS3* transcripts are unequally distributed over the various tissues present in the mature pistil (see Figure 5J for *fbp6*; G.C. Angenent, unpublished data), it is important to compare tissues of the same identity. Therefore, we isolated RNA from ovules present in wild-type plants and the less severe *spaghetti* mutant T27017.

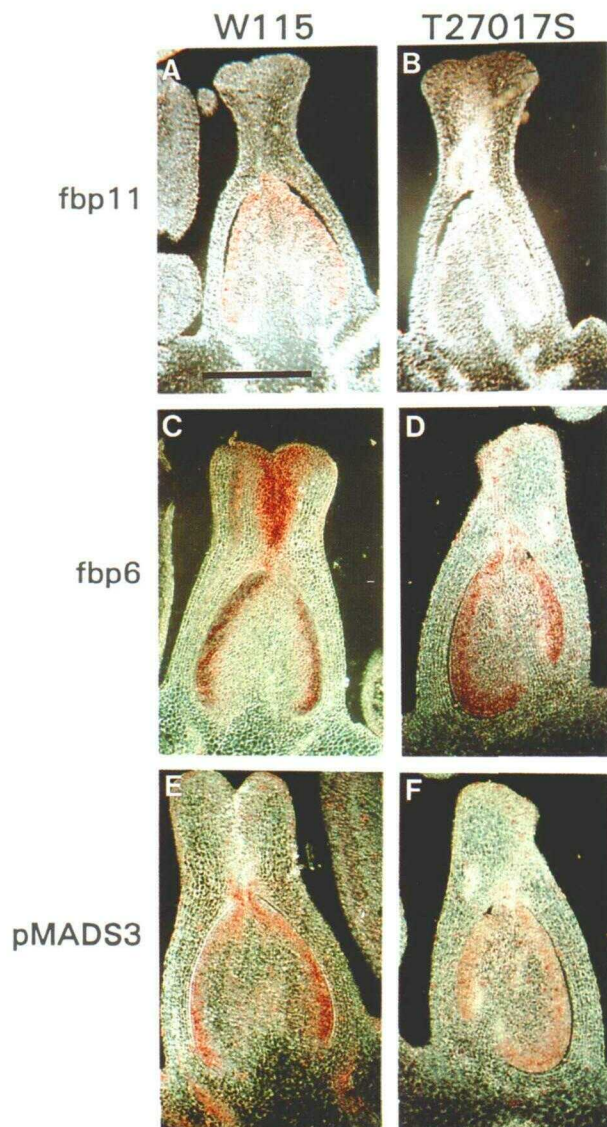


Figure 8. Expression of MADS Box Genes in Developing Ovaries of Wild-Type Plants and *spaghetti* Mutants.

Longitudinal sections from stage 6 pistils were hybridized with digoxigenin-labeled antisense *fbp11*, *fbp6*, and *pMADS3* RNA. All sections were viewed using dark-field microscopy.

(A) Wild-type pistil probed with *fbp11*.

(B) T27017S pistil probed with *fbp11*.

(C) Wild-type pistil probed with *fbp6*.

(D) T27017S pistil probed with *fbp6*.

(E) Wild-type pistil probed with *pMADS3*.

(F) T27017S pistil probed with *pMADS3*.

Bar in (A) = 1 mm.

Because ovules were rare in the severe mutant (T27017S), we isolated RNA from the carpelloid structures and included this RNA in the RNA gel blot as a control. Almost identical levels of *fbp6* and *pMADS3* transcripts were found in both normal ovules and ovules from the primary transformant in which *fbp7* and *fbp11* expression was dramatically reduced. The expression levels of *fbp6* and *pMADS3* in the mature carpelloid outgrowths followed the changes in identity of these structures. *fbp6* accumulation was much higher in the carpelloid structures than in ovules, which is in line with its predominant expression in stylar and stigmatic tissue (see Figures 5J and 8C). The expression of *pMADS3* was also slightly increased in the severe mutant. This fits with the higher accumulation in stylar tissue compared with ovules (G.C. Angenent, unpublished results).

In conclusion, the in situ hybridization experiment and the RNA gel blot analyses showed that despite the suppression of *fbp7* and *fbp11* in the ovule primordia and in ovules of the *spaghetti* mutants, the expression of *fbp6* and *pMADS3* was barely affected, suggesting that these class C genes are not directly controlled by either *fbp7* or *fbp11*.

DISCUSSION

In this study, we describe the isolation of two petunia MADS box genes, *fbp7* and *fbp11*, and the investigation of their role in floral organ development. The MADS box transcription factors encoded by these genes share extensive amino acid sequence homology with each other but not with any of the known MADS box proteins from other species. Expression studies showed that *fbp7* and *fbp11* were coordinately and specifically expressed in ovules, and their mRNAs were already detectable in placental tissue just before the formation of ovule primordia. This expression pattern suggests a regulatory role of these genes in the formation of ovules. This role was confirmed by the homeotic transformation observed in the *spaghetti* mutants.

Ovule Identity Is Changed by Cosuppression of *fbp11*

Cosuppression of the *fbp11* gene in transgenic petunia plants resulted in the homeotic transformation of ovules into carpelloid structures. In several independent primary transformants, a few spaghetti structures were observed in the top part of the ovary, whereas almost all ovules were replaced by carpelloid structures in *spaghetti* mutants homozygous for the cosuppression gene. These carpelloid structures originate directly from the placenta and consist of tissues characteristic of style and stigma. Although at early developmental stages (stage 11), aberrant structures with a chimeric identity were observed, the identity of these structures in mature ovaries was more uniform. Either these structures elongated and developed into carpels, or morphologically normal ovules were

formed. The frequency of ovule conversions seems to be related to the residual *fbp11* expression in the mutants. Low residual gene expression in the primary transformants was sufficient to overcome a certain threshold required for normal ovule development.

Surprisingly, the expression of *fbp7* was also reduced to approximately the same extent as *fbp11*. It might be that the *fbp7* gene is also inhibited by the cosuppression machinery due to the extensive nucleotide sequence homology between *fbp7* and *fbp11*. Alternatively, *fbp11* might regulate *fbp7* gene expression, and consequently, suppression of *fbp11* results in a down-regulation of *fbp7*. The gene products of both *fbp7* and *fbp11* may form heterodimers that autoregulate the transcription of each gene similar to the class B genes in Arabidopsis (Jack et al., 1994), Antirrhinum (Tröbner et al., 1992), and petunia (Angenent et al., 1995). This possible interaction between *fbp7* and *fbp11* and their separate roles in determining ovule identity will be the subject of future studies.

fbp7 and *fbp11* Belong to a New Class of MADS Box Genes

The ABC model describes the action and interactions of three classes of homeotic genes determining the identity of the four floral organs: sepals, petals, stamens, and carpels (for review, see Coen and Meyerowitz, 1991). The evolutionary origin of ovules indicates that they represent distinct organs within the angiosperm and gymnosperm flower (Gifford and Foster, 1989; Angenent et al., 1994) and therefore may be regarded as the fifth organ type in the flower. In this study, we describe two ovule-specific petunia genes that are involved in the determination of ovule identity.

Two different regulatory roles can be postulated for *fbp7* and *fbp11* in specifying the identity of ovules. *fbp7* and *fbp11* might be either cadastral genes setting boundaries for the organ identity genes or organ identity genes specifying ovule identity. Because the final fate of the reversion in the *spaghetti* mutants is as a carpel, whose identity is controlled by the class C homeotic genes, *fbp7* and *fbp11* might be suppressors of C gene expression. In this respect, *fbp7* and *fbp11* prevent the ectopic expression of C-type organ identity genes to allow ovule development rather than a reiteration of carpel organogenesis. It has already been demonstrated that ectopic expression of a C-type gene leads to the formation of carpels at positions normally occupied by ovules. Mandel et al. (1992) reported that ectopic expression of the C-type gene of oilseed rape (*BAG1*) in tobacco results in the transformation of ovules into carpelloid structures. Similarly, expression of AG under the control of the CaMV 35S promoter in transgenic Arabidopsis plants phenocopies these mutants (Ray et al., 1994). In line with this homeotic transformation is the persistent expression of AG in ovules observed in the Arabidopsis *bel1* mutant. In this mutant, the integuments are replaced by carpelloid structures (Modrusan et al., 1994; Ray et al., 1994). The authors suggested that the *BEL1* gene negatively regulates AG late during ovule development.

A similar cadastral function for *fbp7* and *fbp11* might also be the simplest interpretation for the phenotype observed in the *spaghetti* mutant. Therefore, we investigated the expression of two candidates for the petunia C function in mutant and wild-type flowers. They are *fbp6* (Angenent et al., 1993) and *pMADS3* (Tsuchimoto et al., 1993). These petunia genes share extensive sequence homology with AG and are expressed both in whorl three and four organ primordia and at low levels in mature wild-type ovules like AG (G.C. Angenent, unpublished data). Furthermore, ectopic expression of *pMADS3* resulted in a partial conversion of sepals into carpels and petals into anthers, which mimics the *Apetala2* phenotype obtained by ectopic expression of AG (Mizukami and Ma, 1992; Tsuchimoto et al., 1993). Comparison of the expression levels of *fbp6* and *pMADS3* in ovule primordia (stage 6) of wild-type plants and the *spaghetti* mutant revealed that despite the complete down-regulation of *fbp7* and *fbp11*, the expression of both genes was not significantly affected. This suggests that neither *fbp7* nor *fbp11* is a suppressor of C gene expression in wild-type petunia ovules at developmental stages when the identity of the ovules is determined. Also, comparison of C gene expression levels in mature ovules of wild-type plants and primary transformants argues against a cadastral function of *fbp7* and *fbp11*. Only in the spaghetti-shaped structures of the severe mutant were enhanced levels of *fbp6* and *pMADS3* observed; however, this might have been an indirect effect of the changes in tissue identity.

Other observations also support the notion that C gene expression in ovules is not suppressed by *fbp7* and *fbp11*. Accumulation of *fbp7* and *fbp11* transcripts was observed before the formation of ovule primordia at developmental stages when both *fbp6* and *pMADS3* are highly expressed. Furthermore, ectopic expression of *fbp7* or *fbp11* in transgenic plants did not reduce the expression levels of the class C genes (L. Colombo, G.C. Angenent, and A.J. van Tunen, unpublished data). Taken together, these results argue against a function of *fbp7* and *fbp11* as cadastral genes regulating the expression of C-type genes in petunia. More likely, *fbp7* and *fbp11* are organ identity genes inducing developmental pathways leading to the formation of ovules. This is in line with phenotypical alterations observed in transgenic plants in which either the *fbp7* or *fbp11* cDNA was ectopically expressed under the control of the CaMV 35S promoter (L. Colombo, G.C. Angenent, and A.J. van Tunen, unpublished data). Overexpression of these genes resulted in ectopic formation of ovules.

Because both overexpression of the C-type gene and down-regulation of *fbp7* and *fbp11* can affect the identity of ovules, we hypothesize that the balance between C and the ovule-specific genes is crucial for the final fate of the meristem. Excessive C gene overexpression could compete successfully with the normal expression levels of *fbp7* and *fbp11* and subsequently reiterate the program for the formation of carpels instead of ovules.

Whether orthologs of *fbp7* and *fbp11* are present in other plant species is not known. The Arabidopsis *bel1* mutant mimics our *spaghetti* mutant, although in the *bel1* mutant only

part of the ovule is converted into a carpelloid structure, whereas in the *spaghetti* mutant the reversion is complete. This suggests that the *BEL1* gene is acting later during ovule development than are the petunia genes *fbp7* and *fbp11*. In addition, persistent expression of AG in the carpelloid structures of the *bel1* mutant suggests that, in contrast to *fbp7* and *fbp11*, *BEL1* is a cadastral gene regulating AG expression (Ray et al., 1994). Finally, the recent cloning of the *BEL1* gene revealed that this Arabidopsis gene belongs to the homeobox family of transcription factors and is therefore not the homolog of the petunia MADS box genes *fbp7* and *fbp11* (Haughn et al., 1994).

In conclusion, the data presented here indicate that *fbp7* and *fbp11* belong to a new class of MADS box genes that are required for proper ovule development in petunia. Furthermore, our results support the notion that ovules can be regarded as separate organs within the angiosperm flower and may have implications for the evolutionary origin of ovules. Future experiments will determine whether these petunia genes are not only important for the correct formation of ovules but indeed sufficient for the induction of ovule development.

METHODS

Plant Material

Petunia hybrida line W115 and transgenic plants were grown under normal greenhouse conditions.

Screening of cDNA Libraries

A cDNA library was made from poly(A)⁺ RNA of young W115 pistils (±5 mm in length), using the λ-ZAPII vector (Stratagene). Approximately 100,000 plaques were screened with a mixed MADS box probe containing the 5' terminal sequences of *floral binding protein* gene 1 (*fbp1*; 362 bp) and *fbp2* (364 bp) (Angenent et al., 1992). Hybridization and washing of the Hybond N⁺ membranes (Amersham) were done under low-stringency conditions (60°C hybridization and wash with 2 × SSC [1 × SSC is 0.15 M NaCl, 0.015 M sodium citrate] at 60°C). Approximately 150 positive plaques were isolated, from which 75 were analyzed further. From these, 11 independent cDNA clones, designated *fbp2* and *fbp5* to *fbp14*, were obtained. The *fbp14* gene appeared to be identical to the recently isolated petunia gene *pMADS3* (Tsuchimoto et al., 1993). *fbp7* and *fbp11* were each represented by only a single positive plaque (of the 75 screened). Screening of a cDNA library made from poly(A)⁺ from ovules (stage 13 and 14) revealed the same MADS box genes. The pBluescript SK[−] plasmid containing the insert was excised in vivo from the λ-ZAP vector, according to the protocol of Stratagene.

Construction of Binary Vector and Plant Transformation

For cosuppression of *fbp11*, we used the same binary vector used for *fbp1* (Angenent et al., 1993). The *fbp1* cDNA insert was replaced by

the full-length *fbp11* cDNA, using the BamHI (5') and XhoI (3') restriction sites present in the pBluescript SK- vector. The chimeric construct was transferred via *Agrobacterium tumefaciens* LBA4404 into petunia line W115, using the standard leaf disc transformation method (Horsch et al., 1985). Regeneration of the transformants was performed as described by van Tunen et al. (1989).

DNA and RNA Gel Blot Analyses

Total plant DNA was isolated from young petunia leaf material according to Koes et al. (1986), and 10 µg was digested, electrophoresed, and blotted onto Hybond N⁺ membranes. Blots were hybridized as described previously (Angenent et al., 1992) and washed with 0.1 × SSC at 65°C. Total RNA was isolated from leaves or mature floral tissues according to Verwoerd et al. (1989), and 20 µg was denatured by glyoxal (1.5 M) prior to electrophoresis. Gene-specific 3' terminal fragments of *fbp2* (620 bp), *fbp6* (481 bp), *fbp7* (583 bp), *fbp11* (365 bp), and *pMADS3* (635 bp) were labeled by oligonucleotide priming (Feinberg and Vogelstein, 1984). Hybridization was performed as described previously (Angenent et al., 1992). A digoxigenin-labeled actin probe (Boehringer Mannheim) was used, and immunological detection was performed as described in the Boehringer protocol.

RNAse Protection Analysis

³²P-labeled *fbp7* and *fbp11* antisense RNA was synthesized in vitro using T7 polymerase. The in vitro transcription mixture contained 200 ng of plasmid (pBluescript SK- with *fbp7* and *fbp11* cDNA), 1 mM each of GTP, CTP, and ATP, 10 µM UTP, and 10 µCi of ³²P-UTP. Prior to transcription, the plasmid was linearized with EcoRI for *fbp7*, resulting in a 583-base transcript, and with HindIII for *fbp11*, resulting in a 795-base transcript. The labeled transcripts were hybridized with 10 µg of total RNA isolated from petals and ovaries. As a control for the specificity of the RNase treatment, the labeled antisense transcripts were also hybridized with unlabeled *fbp7* and *fbp11* sense transcripts. Sense RNA transcripts were in vitro synthesized using the full-length *fbp7* and *fbp11* cDNAs as templates. ³²P-UTP was replaced by 1 mM UTP in the transcription mixture. Hybridization and RNase digestion were performed as described by van Tunen et al. (1988). Protected fragments were electrophoresed on a 1.2% agarose-formaldehyde gel, blotted onto a nylon membrane, and autoradiographed. The amount of radioactivity in each protected fragment was measured using a PhosphorImager (Molecular Dynamics, Sunnyvale, CA).

In Situ RNA Hybridizations

Floral buds were fixed and embedded in paraffin; sections (10 µm) were made as described by Cañas et al. (1994). Digoxigenin-labeled RNA probes were synthesized by in vitro transcription using the pSPT18/pSPT19 vectors (Boehringer Mannheim). For the synthesis of antisense RNA, 3' terminal cDNA fragments of *fbp6* (481 bp), *fbp7* (583 bp), and *fbp11* (365 bp) were introduced into pSPT18 or pSPT19, using the internal restriction sites HindIII, EcoRI, and PstI, respectively. Transcripts were partly hydrolyzed by incubation at 60°C in 0.1 M Na₂CO₃/NaHCO₃ buffer, pH 10.2, for 30 (*fbp11*), 45 (*fbp6*), and 60 (*fbp7*) min. Hybridization and immunological detection were performed as described by Cañas et al. (1994).

Microscopic Analysis

Ovaries were histochemically stained for β-glucuronidase (GUS) activity according to Koes et al. (1990), and microscopic sections were made as described previously (Angenent et al., 1993).

For the analysis of pollen tube growth on the carpeloid structures of the *spaghetti* mutants, fresh wild-type pollen was loaded onto a partly opened ovary. After 24 hr, the ovary was dissected from the flower and the pollen tubes were analyzed as described previously (Angenent et al., 1993).

For scanning electron microscopy, the ovaries were partly dissected, mounted on a stub, and subsequently frozen in liquid nitrogen. The samples were brought into a cryo-transfer unit (CT 1500 HF; Oxford Instruments, Oxford, UK) and etched for 5 min at -85°C. Subsequently, the sample was sputter coated with 3-nm platinum. The coated specimen was placed inside the scanning electron microscope and observed at 1 to 5 kV. The temperature of the specimen inside the scanning electron microscope was kept at -180°C.

Polymerase Chain Reaction

The presence of the *fbp7* and *fbp11* genes in the progenitors of *P. hybrida* was confirmed by polymerase chain reaction (PCR) using gene-specific primers (ISOGEN Bioscience, Amsterdam). The 3' terminal part of each gene was amplified by one 5' primer (5'-AGGGAGATTCAACTGGAA-CAGG-3'), corresponding to nucleotides 578 to 600 of the *fbp11* cDNA, and specific 3' primers for either *fbp7* (5'-TCCAACTTCATAAGTTCA-ACACC-3') or *fbp11* (5'-TCTTTCTCTCCAGCGCCC-3'). In the PCR mixture, 1 µg of genomic DNA was added as template, and the annealing temperature was 50°C.

ACKNOWLEDGMENTS

We thank Dr. Titti Mariani (Katholieke Universiteit, Nijmegen, The Netherlands) for critical reading of the manuscript and Gerrit Stunnenberg for care of the plants. We also thank Dr. Ronald Koes (Vrije Universiteit, Amsterdam) for providing the ancestor lines of *P. hybrida* and Dr. Adriaan van Aelst for his assistance with the scanning electron microscopy experiments.

Received April 26, 1995; accepted August 15, 1995.

REFERENCES

- Angenent, G.C., Busscher, M., Franken, J., Mol, J.N.M., and van Tunen, A.J. (1992). Differential expression of two MADS box genes in wild-type and mutant petunia flowers. *Plant Cell* 4, 983-993.
- Angenent, G.C., Franken, J., Busscher, M., Colombo, L., and van Tunen, A.J. (1993). Petal and stamen formation in petunia is regulated by the homeotic gene *fbp1*. *Plant J.* 3, 101-112.
- Angenent, G.C., Franken, J., Busscher, M., Weiss, D., and van Tunen, A.J. (1994). Co-suppression of the petunia homeotic gene *fbp2* affects the identity of the generative meristem. *Plant J.* 5, 33-44.

- Angenent, G.C., Busscher, M., Franken, J., Dons, H.J.M., and van Tunen, A.J. (1995). Functional interaction between the homeotic genes *fbp1* and *pMADS1* during petunia floral organogenesis. *Plant Cell* **7**, 507–516.
- Cañas, L.A., Busscher, M., Angenent, G.C., Beltran, J.-P., and van Tunen, A.J. (1994). Nuclear localization of the petunia MADS box protein FBP1. *Plant J.* **6**, 597–604.
- Coen, E.S., and Meyerowitz, E.M. (1991). The war of the whorls: Genetic interactions controlling flower development. *Nature* **353**, 31–37.
- Coen, E.S., Romero, J.M., Doyle, S., Elliott, R., Murphy, G., and Carpenter, R. (1990). *floricaula*: A homeotic gene required for flower development in *Antirrhinum majus*. *Cell* **63**, 1311–1322.
- Davies, B., and Schwarz-Sommer, Z. (1994). Control of floral organ identity by homeotic MADS box transcription factors. In *Results and Problems in Cell Differentiation*, L. Nover, ed (Berlin: Springer-Verlag), pp. 235–258.
- Evans, P.T., and Malmberg, R.L. (1989). Alternative pathways of tobacco placental development: Time and commitment and analysis of a mutant. *Dev. Biol.* **136**, 273–283.
- Feinberg, A.P., and Vogelstein, B. (1984). A technique for radiolabeling DNA restriction endonuclease fragments to high specific activity. *Anal. Biochem.* **137**, 266–267.
- Gifford, E.M., and Foster, A.S. (1989). *Morphology and Evolution of Vascular Plants*. (New York: W.H. Freeman).
- Haughn, G., Modrusan, Z., Samach, A., and Wilkinson, M. (1994). The control of floral morphogenesis in *Arabidopsis*: Regulating the regulators. *Flowering Newslett.* **18**, 12–20.
- Horsch, R.B., Fry, J.E., Hoffman, N.L., Eichholz, D., Rogers, S.G., and Fraley, R.T. (1985). A simple and general method for transferring genes into plants. *Science* **227**, 1229–1231.
- Huijser, P., Klein, J., Lönning, W.-E., Meijer, H., Saedler, H., and Sommer, H. (1992). Bracteomania, an inflorescence anomaly, is caused by the loss of function of the MADS box gene *squamosa* in *Antirrhinum majus*. *EMBO J.* **11**, 1239–1249.
- Jack, T., Fox, G.L., and Meyerowitz, E.M. (1994). *Arabidopsis* homeotic gene *APETAL3* ectopic expression: Transcriptional and posttranscriptional regulation determine floral organ identity. *Cell* **76**, 703–716.
- Koes, R.E., Spelt, C.E., Reif, H.J., van den Elzen, P.J.M., Veltkamp, E., and Mol, J.N.M. (1986). Floral tissue of *Petunia hybrida* (V30) expresses only one member of the chalcone synthase multigene family. *Nucleic Acids Res.* **14**, 5229–5239.
- Koes, R.E., van Blokland, R., Quattrocchio, F., van Tunen, A.J., and Mol, J.N.M. (1990). Chalcone synthase promoters in petunia are active in pigmented and unpigmented cell types. *Plant Cell* **2**, 379–392.
- Ma, H., Yanofsky, M.F., and Meyerowitz, E.M. (1991). *AGL1-AGL6*, an *Arabidopsis* gene family with similarity to floral homeotic and transcription factor genes. *Genes Dev.* **5**, 484–495.
- Mandel, M.A., Bowman, J.L., Kempin, S.A., Ma, H., Meyerowitz, E.M., and Yanofsky, M.F. (1992). Manipulation of flower structure in transgenic tobacco. *Cell* **71**, 133–143.
- Mizukami, Y., and Ma, H. (1992). Ectopic expression of the floral homeotic gene *AGAMOUS* in transgenic *Arabidopsis* plants alters floral organ identity. *Cell* **71**, 119–131.
- Modrusan, Z., Reiser, L., Feldmann, K.A., Fischer, R.L., and Haughn, G.W. (1994). Homeotic transformation of ovules into carpel-like structures in *Arabidopsis*. *Plant Cell* **6**, 333–349.
- Ray, A., Robinson-Beers, K., Ray, S., Baker, S.C., Lang, J.D., Preuss, D., Milligan, S.B., and Gasser, C.S. (1994). *Arabidopsis* floral homeotic gene *BELL* (*BEL1*) controls ovule development through negative regulation of *AGAMOUS* gene (*AG*). *Proc. Natl. Acad. Sci. USA* **91**, 5761–5765.
- Reiser, L., and Fischer, R.L. (1993). The ovule and the embryo sac. *Plant Cell* **5**, 1291–1301.
- Schwarz-Sommer, Z., Huijser, P., Nacken, W., Saedler, H., and Sommer, H. (1990). Genetic control of flower development by homeotic genes in *Antirrhinum majus*. *Science* **250**, 931–936.
- Tröbner, W., Ramirez, L., Motte, P., Hue, I., Huijser, P., Lönning, W., Saedler, H., Sommer, H., and Schwarz-Sommer, Z. (1992). *globosa*: A homeotic gene which interacts with *deficiens* in the control of *Antirrhinum* floral organogenesis. *EMBO J.* **11**, 4693–4704.
- Tsuchimoto, S., van der Krol, A.R., and Chua, N.-H. (1993). Ectopic expression of *pMADS3* in transgenic petunia phenocopies the petunia *blind* mutant. *Plant Cell* **5**, 843–853.
- van der Krol, A.R., Mur, L.A., Beld, M., Mol, J.N.M., and Stuitje, A.R. (1990). Flavonoid genes in petunia: Addition of a limited number of gene copies may lead to a suppression of gene expression. *Plant Cell* **2**, 291–299.
- van Tunen, A.J., Koes, R.E., Spelt, C.E., van der Krol, A.R., and Mol, J.N.M. (1988). Cloning of the two chalcone flavanone isomerase genes from *Petunia hybrida*: Coordinate, light-regulated and differential expression of flavonoid genes. *EMBO J.* **7**, 1257–1263.
- van Tunen, A.J., Mur, L.A., Brouns, G.S., Rienstra, J.-D., Koes, R.E., and Mol, J.N.M. (1989). Pollen- and anther-specific *chi* promoters from petunia: Tandem promoter regulation of the *chiA* gene. *Plant Cell* **2**, 393–401.
- Verbeke, J.A. (1992). Fusion events during floral morphogenesis. *Annu. Rev. Plant Physiol. Plant Mol. Biol.* **43**, 583–598.
- Verwoerd, T.C., Dekker, B.M.M., and Hoekema, A. (1989). A small-scale procedure for the rapid isolation of plant RNAs. *Nucleic Acids Res.* **17**, 2362.
- Weigel, D., and Meyerowitz, E.M. (1994). The ABCs of floral homeotic genes. *Cell* **78**, 203–209.
- Weigel, D., Alvarez, J., Smyth, D.R., Yanofsky, M.F., and Meyerowitz, E.M. (1992). *LEAFY* controls floral meristem identity in *Arabidopsis*. *Cell* **69**, 843–859.
- Willemsse, M.T.M., and van Went, J.L. (1984). The female gametophyte. In *Embryology of Angiosperms*, B.M. Johri, ed (Berlin: Springer-Verlag), pp. 159–196.
- Ylstra, B., Busscher, J., Franken, J., Hollman, P.C.H., Mol, J.N.M., and van Tunen, A.J. (1994). Flavonols and fertilization in *P. hybrida*: Localization and mode of action during pollen tube growth. *Plant J.* **6**, 201–212.